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Genotype Analyses of *Campylobacter* Isolated from Distinct Segments of the Reproductive Tracts of Broiler Breeder Hens

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Abstract. *Campylobacter* isolated from feces and from the oviduct of six broiler breeder hens were genotyped by using *flaA* SVR DNA sequence analyses. A diversity of genotypes was observed among fecal and oviduct isolates. Comparison of isolates from the oviducts of individual hens revealed variable results. In three cases (hen 2, hen 3, and hen 6), analyses indicated that isolates from all regions of the individual hen's reproductive tract were closely related; isolates from hen 1 and hen 4 were diverse. Comparison of the *Campylobacter* isolates between hens revealed that in two cases, hens 1 and 3 and hens 4 and 6, certain isolates possessed identical *flaA* SVR sequence types. Comparisons of *Campylobacter* isolates recovered from a distinct region of the oviduct were found to have increased diversity as sampling progressed down the oviduct. This study further demonstrates that *Campylobacter* is present within the reproductive tract of breeder hens and that this presence may enable vertical transmission of *Campylobacter* from the breeder hen to the broiler offspring.

Campylobacter, a Gram-negative, microaerophilic bacterium, is presently believed to be the leading bacterial etiological agent of acute gastroenteritis in the human population; the total number of *Campylobacter* enteritis cases in the United States is estimated at 2.4 million per year, or approximately 1–2% of the population per year [1, 22, 26, 27]. *Campylobacter* infections have also been associated with unnecessary appendectomies, reactive arthritis, and the development of Guillain-Barré syndrome, although these complications are infrequent [2–4, 29]. Mishandling and consumption of inadequately cooked poultry or poultry products are considered to be primary sources for *Campylobacter*-induced disease in humans [3, 16, 19]. *Campylobacter* has been cultured from as many as 75% of the live broiler population and from as much as 80% of processed poultry meat samples sold commercially [10–12, 21]. The high colonization incidence of poultry and the resultant clinical infections in humans have prompted a number of investigations focused upon identifying and subsequently eliminating sources of *Campylobacter* contamination in chickens. However, the pathways involved in *Campylobacter* contamination of poultry flocks, horizontal transmission and/or vertical transmission, continue to remain unclear. Several suspected sources or vectors for *Campylobacter* contamination have been investigated and include environment of the poultry house, hatchery pads, litter, feed, water, personnel, small animals on the farm, flies, and rodents [9, 13, 15, 24, 26].

Recently, evidence has emerged that implicates breeder hens as a potential source for *Campylobacter* contamination of the subsequent broiler offspring. Genotype analyses of *Campylobacter* isolated from commercial broiler breeder flocks and from the respective broiler progeny demonstrated that the isolates from these epidemiologically related sources were clonal in origin [8]. Additionally, *Campylobacter* was isolated from the oviducts of laying hens; this presence in the oviduct was thought to result from ascending infection via the cloaca [5]. Further evidence supporting breeder/broiler transmission was the demonstration that, while *Campylobacter* was not detected by traditional cultural methods in hatchery debris, PCR was capable of detecting the

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Table 1. Sources of *Campylobacter* oviduct isolates investigated[a]

Hen	Reproductive tract segment					
	Magnum	Isthmus	Shell gland	Vagina	Cloaca	Totals
1	1	0	1	1	1	4
2	1	2	2	2	1	8
3	1	0	1	1	2	5
4	0	0	1	1	1	3
5	0	0	0	0	0	0
6	0	0	1	0	1	2
Totals	3	2	6	5	6	22

[a] The 20 fecal isolates analyzed in this study are not included in the table.

presence of *Campylobacter* DNA in these hatchery samples [10]. In an effort to further understand the role of the breeder reproductive tract in transmission of *Campylobacter* to the broiler offspring, *Campylobacter* isolated from feces and from distinct segments of the reproductive tracts (magnum, isthmus, shell gland, vagina, and cloaca) of six broiler breeder hens were genotyped by using *flaA* SVR DNA sequence analyses.

Materials and Methods

Bacterial isolates. *Campylobacter* isolates used in this study are described in Table 1. Fresh fecal droppings from six individually caged commercial breeder hens were aseptically collected into sterile tubes and packed in crushed ice prior to transport to the laboratory. Each sample was weighed and diluted 1:3 (wt/vol) with phosphate-buffered saline (PBS). Serial dilutions were prepared and plated onto Campy-Cefex agar, which was incubated at 42°C for 36–48 h in microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) [23]. After incubation, a representative number of presumptive *Campylobacter* colonies were confirmed by observation of typical cellular morphology with phase contrast microscopy and with a commercial latex agglutination kit.

Reproductive tract isolates were obtained from 61-week-old commercial breeder hens that previously tested positive for *Campylobacter* by fecal sampling. After the hens were scalded and defeathered, individual reproductive tracts were aseptically excised and divided into the following segments: magnum, isthmus, shell gland, vagina, and cloaca. Each segment was placed into a sterile plastic bag, suspended 1:3 (wt/vol) in Bolton's enrichment broth, and homogenized for 1 min. One hundred microliters of suspension was removed, direct-plated onto Campy-Cefex agar, and incubated as above. The remaining suspension was enriched at 42°C for 48 h followed by direct plating onto Campy-Cefex agar. Plates were incubated, and presumptive *Campylobacter* colonies were confirmed as previously described. All *Campylobacter* cultures were stored in Brucella broth containing glycerol (16% [wt/vol]) and frozen at −80°C.

***flaA* SVR DNA sequence analyses.** Isolated colonies of *Campylobacter* were resuspended in 300 μl of sterile H_2O and placed at 100°C for 10 min. Ten microliters of each boiled cell suspension was used as template for *flaA* SVR PCR with the following primers: FLA242FU: $^{5'}$CTA TGG ATG AGC AAT TWA AAA T$^{3'}$ and FLA625RU: $^{5'}$CAA GWC CTG TTC CWA CTG AAG$^{3'}$ [18]. A 35-cycle reaction was used with 1 min denaturing at 96°C, 1 min annealing at 52°C, and a 1-min extension at 72°C. The resulting product was approximately 425 bp. Sequence data were generated by using either the FLA242FU primer or the FLA625RU primer with the Big-Dye Dye-Terminator Cycle Sequencing Kit (ABI-PE, Foster City, CA). Data were assembled with Sequencher 4.1 (GeneCodes Corp., Ann Arbor, MI) and aligned with ClustalIX [28]. Aligned sequences were compared, and dendograms were generated by using the UPGMA algorithm with HKY85 distance measurements in PAUP*4.0 (Phylogentic Analysis Using Parsimony) [25].

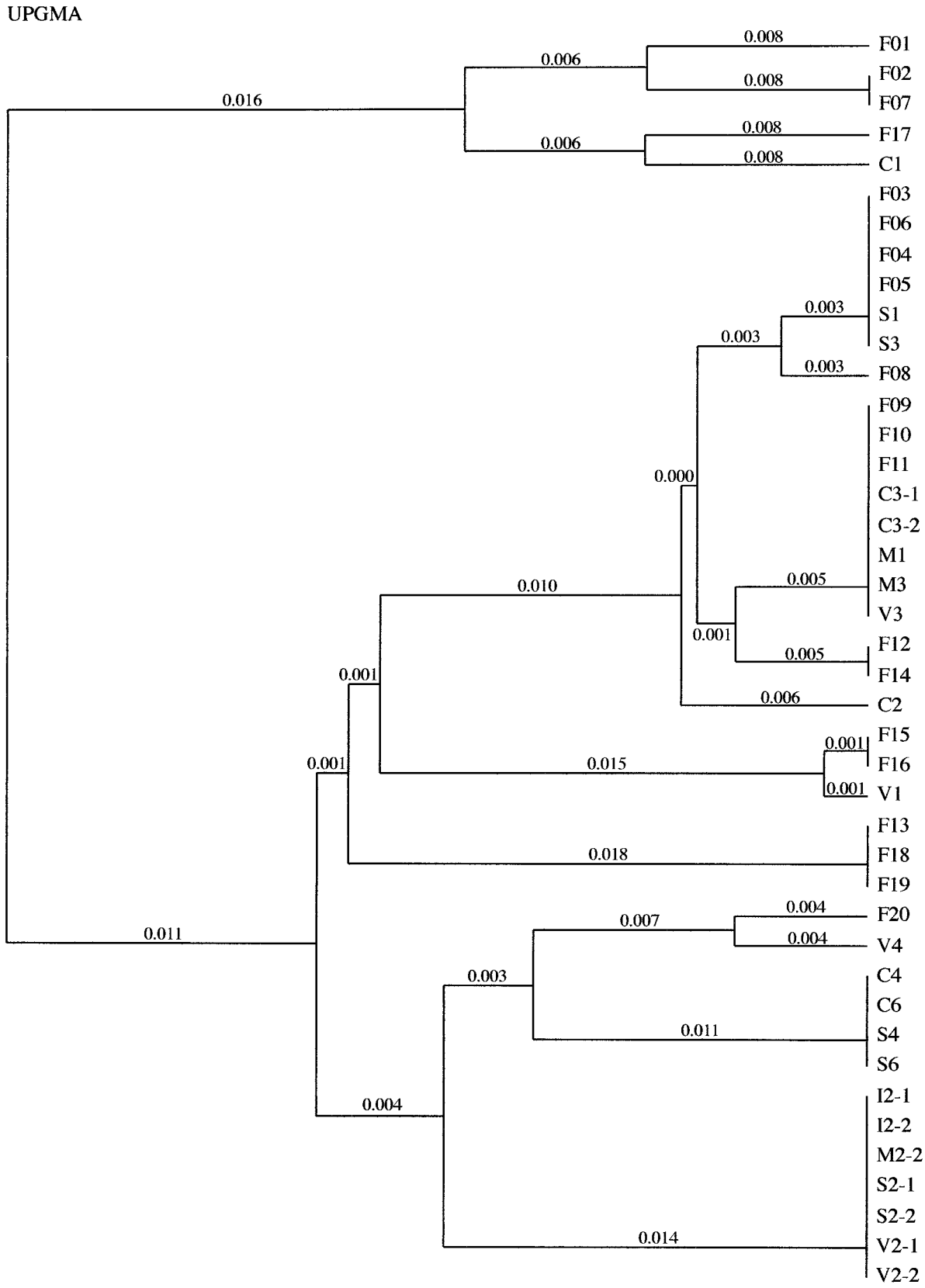
Results

A total of 42 *Campylobacter* isolates were analyzed in this study; 20 isolates originated from feces of the breeder hens, and 22 isolates were from oviducts. Information on the reproductive tract isolates is shown in Table 1. *Campylobacter* were detected (by traditional cultural methods) within the reproductive tract of five of the six breeder hens tested. In general, the numbers of *Campylobacter* increased as sampling progressed from the initial segments of the reproductive tract (magnum and isthmus) toward the terminal segments of the reproductive tract (shell gland, vagina, and cloaca).

DNA sequence analysis revealed that a diversity of sequence types was present among fecal isolates and among oviduct isolates. A dendogram containing all isolates analyzed in this study is presented in Fig. 1. Fecal isolates demonstrated as much as 7.2% variability. Reproductive tract isolates demonstrated up to 6.2% variability. Comparison of fecal and reproductive tract isolates revealed 7.2% variability, the same as for the fecal isolates alone. Of the 22 oviduct isolates analyzed, 8 possessed *flaA* SVR DNA sequences identical to several fecal isolates. All of the oviduct isolates from hen 3 matched fecal isolates; four isolates, two cloacal isolates, one magnum isolate, and one vaginal isolate matched fecal isolates F09, F10, and F11. The remaining isolate from hen 3, a shell gland isolate, matched fecal isolates F03, F04, F05, and F06. Three isolates from hen 1 matched certain fecal isolates: a shell gland isolate matched fecal isolates F03, F04, F05, and F06. A magnum isolate matched three fecal isolates F09, F10, and F11, and a vaginal isolate matched fecal isolates F15 and F16.

One of the oviduct isolates, a vaginal isolate from hen 4, was considered to be closely related to fecal isolate F20 (0.894% difference) and, most likely, clonal in origin. The remaining 13 oviduct isolates were found to be distantly related to all fecal isolates collected. Variability of these oviduct isolates to fecal isolates ranged from 1.79% to 6.56%. Interestingly, this group included all of the isolates gathered from hen 2 and from hen 6.

Comparison of oviduct isolates collected from


UPGMA
0.008
F01
0.006
0.008
F02
0.016
F07
0.008
F17
0.006
0.008
C1
F03
F06
F04
F05
0.003
S1
0.003
S3
0.003
F08
F09
F10
F11
0.000
C3-1
C3-2
M1
0.010
0.005
M3
V3
0.001
F12
0.005
F14
0.001
0.006
C2
F15
0.001
0.001
0.015
F16
0.001
V1
F13
0.018
F18
F19
0.004
0.007
F20
0.011
0.004
V4
0.003
C4
C6
0.011
S4
S6
0.004
I2-1
I2-2
M2-2
S2-1
S2-2
0.014
V2-1
V2-2

within individual hens revealed variable results. Three of the hens tested possessed isolates in which all or a majority of the isolates were identical or closely related regardless of the location within the oviduct. In hen 6, the two isolates recovered, one shell gland and one cloaca, were genotypically identical by *flaA* SVR DNA sequence analysis. Seven of the eight isolates collected from hen 2 were also genotypically identical; the remaining cloacal isolate was distantly related (3.65% difference) to all other hen 2 isolates. Of the five isolates collected from hen 3, four were genotypically identical, with the remaining isolate differing by only 0.894%. Isolates from hen 1 and hen 4 demonstrated increased diversity compared with hens 2, 3, and 6; variabilities of as much as 6.21% and 2.10%, respectively, were observed.

Comparison of the *Campylobacter* isolates between hens revealed that, in two cases, hens 1 and 3 and hens 4 and 6, certain isolates possessed identical *flaA* SVR sequence types (Fig. 1). Isolates cultured from hens 4 and 6 (shell gland and cloacal isolates) possessed identical genotypes, while the isolates recovered from the cloaca, magnum, and vagina of hen 3 were similar to a magnum isolate from hen 1. Additionally, shell gland isolates from both hen 1 and hen 3 were identical by *flaA* SVR DNA sequence analysis. Comparisons of *Campylobacter* isolates recovered from a distinct region of the reproductive tract revealed distances of as much as 0.00%, 3.97%, 3.65%, 4.27%, and 5.64% for the magnum, isthmus, shell gland, vagina, and cloaca, respectively.

Discussion

The Centers for Disease Control and Prevention estimate that *Campylobacter* enteritis is a multi-billion-dollar disease and that the consumption of poultry is a primary source of the resultant clinical infections in humans. An understanding of the pathways involved in *Campylobacter* contamination of poultry flocks is, therefore, essential for the development of intervention strategies and the subsequent reduction of *Campylobacter* in poultry. *Campylobacter* colonization of broiler flocks is presumed to originate primarily from a combination of animal sources: (a) farm animals other than broilers present on the broiler farm, (b) farm animal sources outside the broiler farm, (c) domestic pets and vermin, and (d) previous flocks [9, 14, 15, 17, 20]. Accordingly, intervention strategies have focused primarily on control of *Campylobacter* at the farm, or at slaughter; nevertheless, *Campylobacter* contamination of poultry flocks and of the final market product remains a problem.

Recent investigations have identified a new potential source of *Campylobacter* contamination of broiler flocks: transmission from the breeder hens to the broiler offspring via the contaminated fertilized egg [6–8, 10, 13, 17, 20]. In this study, *Campylobacter* was isolated from the oviducts of five of six breeder hens tested. Overall, the numbers of *Campylobacter* recovered were reduced as sampling progressed from the terminal segments of the reproductive tract (cloaca, vagina, and shell gland) toward the initial segments of the reproductive tract (isthmus and magnum). This finding supports the idea that the reproductive tract may become contaminated by reverse peristalsis of *Campylobacter* from the cloaca up through the oviduct. Alternatively, however, the observation that 13 of the 22 oviduct isolates were distantly related to fecal isolates suggests that, although contamination of the oviduct may be through fecal contamination, some isolates of *Campylobacter* may enter the oviduct by other unknown routes.

With respect to comparison of isolates collected from within an individual hen, the data presented are inconclusive. Genotype data from hen 1 and hen 4 show that multiple clones of *Campylobacter* can be present within the oviduct of an individual hen. However, the data from hen 2, hen 3, and hen 6 suggest that only one predominant clone may be present throughout a hen's reproductive tract. Comparisons of *Campylobacter* isolates recovered from distinct regions of the oviduct revealed distances of as much as 0.00%, 3.97%, 3.65%, 4.27%, and 5.64% for the magnum, isthmus, shell gland, vagina, and cloaca, respectively. These data show that as sampling progresses down through the oviduct, the variability of clones increases. This observation may be because fewer *Campylobacter* are in the initial segments of the oviduct; fewer isolates may result in less diversity. An alternative explanation is that some *Campylobacter* may be better adapted than others for colonization of different regions of the reproductive tract.

The findings in this study demonstrate that *Campylobacter* is indeed present within the reproductive tract of breeder hens and that this presence may allow vertical transmission of *Campylobacter* from the breeder hen to

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Fig. 1. Relationships derived from comparison of the SVR (Short Variable Region) of the *flaA* gene from *Campylobacter*. The dendrogram was generated by using the UPGMA algorithm with HKY85 distance measurements in PAUP*4.0 (Phylogenetic Analysis Using Parsimony). Fecal isolates are labeled by F and the corresponding sample number. Reproductive tract isolates are labeled by the location in which they were collected in the oviduct (M = magnum, I = isthmus, S = shell gland, V = vagina, and C = cloaca), followed by the identification of the individual hen.

the broiler offspring. Additional studies are needed to further elucidate the mechanisms by which *Campylobacter* colonization occurs within the oviduct of the breeder hen. Moreover, intervention strategies will have to be developed to aggressively target locations that were previously excluded, such as breeder flocks, hatching cabinets, and hatchery environments. Information such as that presented in this report is necessary to provide a basis for refining or adjusting intervention strategies to produce safer poultry food products, thereby reducing the risk of human exposure.

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